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Pathway of phytate dephosphorylation by \(\beta\)-propeller phytases of different origins

Ralf Greiner, Boon L. Lim, Chiwai Cheng, and Nils-Gunnar Carlsson

Abstract: Using a combination of high-performance ion chromatography analysis and kinetic studies, the pathway of myo-inositol hexakisphosphate dephosphorylation by the \(\beta\)-propeller phyrase of Shewanella oneidensis was established, which was then compared with that of Bacillus subtilis, Bacillus amyloliquefaciens ATCC 15841, and R. amyloliquefaciens 45 \(\beta\)-propeller phytases. The data demonstrate that all of these \(\beta\)-propeller phytases dephosphorylate myo-inositol hexakisphosphate in a stereospecific way by sequential removal of phosphate groups via \(d\)-Ins(1,2,4,5,6)P\(_5\), Ins(2,4,5,6)P\(_4\) to finally Ins(2,4,6)P\(_3\). Thus, the \(\beta\)-propeller phytases prefer the hydrolysis of every second phosphate over that of adjacent ones. This finding does not support previous phytate degradation models proposed by J. Kerovuo, J. Rouvinen, and F. Hatzack (2000. Biochem. J. 352: 623–628) and R. Greiner, A. Farouk, M. Larsson Alminger, and N.G. Carlsson (2002. Can. J. Microbiol. 48: 986–994), but seems to fit with the structural model given by S. Shin, N.C. Ha, B.C. Oh, T.K. Oh, and B.H. Oh (2001. Structure, 9: 851–858).

Key words: Bacillus spp., \(\beta\)-propeller phyrase, myo-inositol phosphate isomers, phytate degradation, Shewanella oneidensis.


Mots-clés : Bacillus spp., phyrase \(\beta\)-hélicoïdale, isomères du myo-inositol phosphate, dégradation du phytate, Shewanella oneidensis.

[Intaduit par la Rédaction]

Introduction

Phytases play a crucial role in the recycling of phytate-phosphorus in the biosphere. Based on their amino acid sequence identity and catalytic mechanisms, phytases can be grouped into four classes: histidine acid phosphatase (HAP), purple acid phosphatase (PAP), \(\beta\)-propeller phytase (BPP), and cysteine phytase (CP) (Mullaney and Ullah 2005). Among these phytases, the BPP family is the only class that exhibits phytase activity at neutral and alkaline pH. Phytases that are constituted by a single BPP domain were previously characterized from several Gram-positive Bacillus strains (Kerovuo et al. 2000; Tye et al. 2002). The three-dimensional structure of the BPP from Bacillus amyloliquefaciens resembles a six-bladed propeller that is mainly composed of highly curved antiparallel \(\beta\)-sheets (Ha et al. 2000). Crucial amino acid residues that are involved in the conformation of two phosphate binding sites and six calcium binding sites are also revealed by the structure. Three of the calcium binding sites are high-affinity binding sites that are responsible for thermostability, and the other three are low-affinity binding sites that are responsible for catalytic activity (Ha et al. 2000).

Recently, a protein (PhyS) with two BPP domains was characterized from the Gram-negative bacterium Shewanella oneidensis (Cheng and Lim 2006). While the five-bladed N-terminal BPP domain showed no phytase activity, the C-terminal six-bladed BPP domain was shown to exhibit highly specific activity towards phytate. Although this catalytic domain shares only 34%–36% sequence identity with Bacillus phytases, PhyS also exhibits calcium-dependent enzymatic activity at neutral to alkaline pH. Genome sequence analysis of the NCBI database and the Sargasso sea environmental database (Venter et al. 2004)
indicate that BPP-like sequences are widely distributed in the genomes of a number of bacteria (Cheng and Lim 2006). While these BPP-like sequences only share low protein sequence identity (20%–40%) with that of PhyS and Bacillus phytases, most of the essential amino acids that are responsible for calcium and phosphate binding are conserved in these sequences (Ha et al. 2000).

One of the objectives of this study was to delineate the phytate-hydrolysis pathway of PhyS and to identify the intermediates as well as the end product. In addition, the phytate degradation pathway of PhyS was compared with that of Bacillus phytases. The phytate-hydrolysis pathways of Bacillus phytases have been experimentally determined by Kerovuo et al. (2000) and Greiner et al. (2002) (Fig. 1). However, discrepancies exist between the two pathways and the computer-generated structural model of BPPs reported by Shin et al. (2001). This could be due to the presence of at least one contaminating phosphatase in the previous enzyme preparations. In this study, a higher quality enzyme preparation was employed and the new data are consistent with the proposed phytate-binding site in the structural model (Shin et al. 2001).

Materials and methods

Chemicals
Aspergillus niger phytase was obtained from Novo Nordisk (Copenhagen, Denmark). Phytic acid dodecasodium salt was from Aldrich (Steinheim, Germany). Ultrasep™ ES 100 RP18 was purchased from Bischoff (Leonberg, Germany) and the high-pressure ion-pair chromatography (HPIC) column Carbo-Pac™ PA-100 was from Dionex (Sunnyvale, California, USA). AG1 X-4, 100–200 mesh resin was obtained from Bio-Rad (Munich, Germany). The source of the myo-inositol phosphate standards were as indicated by Skoglund et al. (1998).

Purification of the phytate-degrading enzymes
Purification of the phytate-degrading enzymes of A. niger (Greiner et al. 2001), Escherichia coli (Greiner et al. 1993), S. oneidensis (Cheng and Lim 2006), and Klebsiella terrigena (Greiner et al. 1997) was performed as described previously.

The encoding genes of the phytases from the different Bacillus strains were inserted between the NdeI and HindIII sites of pET-22b(+)(Novagen, San Diego, California, USA) and over-expressed in E. coli BL21 (DE3). To lyse bacteria, cells were frozen at –80 °C for 10 min and thawed at room temperature for 20 min; freezing and thawing were repeated twice. After the final thaw, bacteria were sonicated for two 1 min intervals. Cell debris was removed by centrifugation at 10 000 g, 4 °C, 30 min and phytases were purified from the clear supernatant by affinity chromatography using Ni-NT agarose (Qiagen, Valencia, California).

All phytases were purified to apparent homogeneity according to denaturing and nondenaturing polyacrylamide gel electrophoresis (data not shown).

Buffers
The following buffers were used for incubating myo-inositol phosphate esters with the phytases: 0.1 mol/L sodium acetate, pH 4.5 (E. coli), 0.1 mol/L sodium acetate, pH 5.0 (A. niger, K. terrigena), 0.1 mol/L Tris–HCl, 2 mmol/L calcium chloride, pH 7.5 (B. subtilis 168, B. amyloliquefaciens ATCC 15841, B. amyloliquefaciens 45), 0.1 mol/L Tris–maleate, and 4 mmol/L calcium chloride, pH 6.0 (S. oneidensis).

Assay of phytase activity
Phytase activity measurements were carried out at 37 °C. The enzymatic reactions were started by the addition of 10 μL of enzyme to the assay mixtures. The incubation mixture for phytase activity determination consisted of 350 μL of incubation buffer containing 500 nmol of sodium phytate. After an incubation time of 30 min, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (Heinonen and Lahti 1981).

Heat treatment of Bacillus phytase preparations
The Bacillus phytase preparations obtained after purification were incubated at 80 °C and 100 °C in the presence and absence of 5 mmol/L calcium for 10 min, cooled to 4 °C, and assayed using the standard phytase assay. To determine their residual activities toward the other phosphorylated compounds, phytate was substituted by D-Ins(1,2,4,5,6)P5, pyrophosphate, and ATP, respectively.
Fig. 2. High-pressure ion-pair chromatography analysis of the enzymatically formed hydrolysis products of myo-inositol hexakisphosphate by the purified phytases from *Shewanella oneidensis* (A) and *Bacillus amyloliquefaciens* ATCC 15841 (B). First profile in each panel corresponds to a reference sample containing a mixture of myo-inositol phosphate standards. Peaks were identified as indicated in Skoglund et al. (1998). Peaks: A, Ins(1,3,4,5,6)P$_5$; B, d/l-Ins(1,2,3,4,5)P$_5$; C, Ins(1,2,3,4,6)P$_5$; D, d/l-Ins(1,4,5,6)P$_5$; E, d/l-Ins(1,2,5,6)P$_5$; G, d/l-Ins(1,3,4,5)P$_5$; H, d/l-Ins(1,2,4,5)P$_5$; K, Ins(1,3,4,6)P$_5$; L, d/l-Ins(1,2,3,4)P$_5$; M, d/l-Ins(1,2,3,5)P$_5$; O, Ins(4,5,6)P$_3$; P, d/l-Ins(1,5,6)P$_3$; R, d/l-Ins(1,4,5)P$_3$; S, d/l-Ins(1,2,6)P$_3$, Ins(1,2,3)P$_3$; T, d/l-Ins(1,3,4)P$_3$; U, d/l-Ins(2,4)P$_2$; X, d/l-Ins(1,2,5)P$_2$, Ins(2,5)P$_2$, d/l-Ins(4,5)P$_2$; and Y, d/l-Ins(1,4)P$_2$, d/l-Ins(1,6)P$_2$. 

(A) 

profile of standards

incubation time: 0 min

incubation time: 30 min

incubation time: 60 min

incubation time: 90 min
Fig. 2 (continued).

![Profile of standards](image)

**Greiner et al.** 2007 NRC Canada
Preparation of myo-inositol pentakis- and tris-phosphate isomers

myo-Inositol hexakisphosphate (50 μmol) in the corresponding incubation buffer were incubated at 37 °C with 0.4 U of the phytases in a final volume of 20 mL. After an incubation period of 60 min (myo-inositol pentakisphosphate preparation) or 8 h (myo-inositol trisphosphate preparation), the reactions were stopped by heat treatment (95 °C, 10 min). The incubation mixtures were lyophilized and the dry residues were dissolved in 10 mL of 0.2 mol/L ammonium formate, pH 2.5. The solutions were loaded onto a Q-Sepharose column (2.6 cm × 90 cm) equilibrated with 0.2 mol/L ammonium formate, pH 2.5, at a flow rate of 2.5 mL/min. The column was washed with 500 mL of 0.2 mol/L ammonium formate, pH 2.5; the bound myo-inositol trisphosphates were eluted with a linear gradient from 0.2 to 0.6 mol/L ammonium formate, pH 2.5 (1 L) and the bound myo-inositol pentakisphosphates with a linear gradient from 1.0 to 1.4 mol/L ammonium formate, pH 2.5 (1000 mL) at 2.5 mL/min. Fractions of 10 mL were collected. From even-numbered tubes, 100 μL aliquots were lyophilized. The residues were dissolved in 1.5 mmol/L sulfuric acid and incubated for 90 min at 165 °C to hydrolyze the eluted myo-inositol phosphates completely. The liberated phosphate was quantified by a modification of the ammonium molybdate method (Heinonen and Lahti 1981). The content of the fraction tubes corresponding to the myo-inositol tris- and pentakis-phosphates, respectively, were pooled and lyophilized until only a dry residue remained. Ten milliliters of water was used to redissolve the residues. Lyophilization and redissolving were repeated twice to completely remove ammonium formate, myo-inositol phosphate concentrations were determined by high-pressure liquid chromatography (HPLC) HPIC on Ultrasep™ ES 100 RP18 (2 mm × 250 mm). The column was run at 45 °C and 0.2 mL/min of an eluant consisting of formic acid–methanol–water–TBAH (tetra-butylammonium hydroxide) (44:56:5:1.5 by volume), pH 4.25, as described by Sandberg and Adhérinne (1986). A mixture of the individual myo-inositol phosphate esters (IP₃–IP₇) was used as a standard. The purity of the myo-inositol phosphate preparations was determined on a HPIC system as described by Skoglund et al. (1998).

Production of enzymically formed hydrolysis products

The enzymatic reaction was started at 37 °C by addition of 50 μL of the suitably diluted solution of the phytases from S. oneidensis or from different Bacillus spp. to the incubation mixtures (100 nM/mL). The incubation mixture consisted of either 1250 μL of 0.1 mol/L Tris–maleate, pH 6.0 containing 5 μmol CaCl₂ and 1.25 mmol sodium phytate (S. oneidensis) or 1250 μL of 0.1 mol/L Tris–HCl, pH 7.5 containing 2.5 μmol CaCl₂ and 2.5 μmol sodium phytate (Bacillus sp.). From the incubation mixture, 150 μL samples were removed periodically and the reaction was stopped by heat treatment (90 °C, 5 min). For the identification of phytate degradation products, 50 μL of the heat-treated samples were chromatographed on a HPIC system as described by Skoglund et al. (1998).

Identification of enzymatically formed hydrolysis products

Isomers of myo-inositol phosphate were determined and separated on a HPIC system using a Carbo Pac PA-100 (4 mm × 250 mm) analytical column and a gradient of 5%–98% HCl (0.5 mol/L, 0.8 mL/min) as described by Skoglund et al. (1998). The eluants were mixed in a post-column reactor with 0.1% (Fe(NO₃)₃)·9H₂O in a 2% (v/v) HClO₄ solution (0.4 mL/min), according to Phillippy and Bland (1988). The combined flow rate was 1.2 mL/min.

Kinetic studies with the pure myo-inositol phosphate isomers

In addition to phytate, the following myo-inositol phosphate isomers were used as substrates for Kₘ and kₘₐₜ determination: d-Ins(1,2,4,5,6)P₇ (product of A. niger phytase), the myo-inositol pentakisphosphates and myo-inositol trisphosphates produced by the phytases from S. oneidensis, B. subtilis 168, B. amyloliquefaciens ATCC 15841, and B. amyloliquefaciens 45. The incubation mixture consisted of 350 μL incubation buffer containing the phosphorylated compound in a serial dilution of a concentrated stock solution (2.0 mmol/L). The enzymatic reactions were started by adding 10 μL of the phytases to the assay mixtures. After an incubation period of 30 min, the liberated phosphate was quantified by a modification of the ammonium molybdate method (Heinonen and Lahti 1981). The rate of reaction was linear for the 30 min incubation time (data not shown). Activity (U) was expressed as μmol phosphate liberated per min. Blanks were run by the addition of ammonium molybdate solution prior to the addition of enzyme solution to the assay mixtures. The kinetic constants (Kₘ, vₘₐₓ) were calculated from the Lineweaver–Burk plots of the data. For the calculation of kₘₐₓ, the following molecular masses were used: 40 kDa for the Bacillus phytases (Greiner et al. 2002) and 73 kDa for the S. oneidensis phytase (Cheng and Lim 2006).

Quantification of liberated phosphate

The liberated phosphate was quantified by a modification of the ammonium molybdate method (Heinonen and Lahti 1981). A 1.5 mL volume of a freshly prepared solution of acetic–5 mol/L sulfuric acid–10 mmol/L ammonium molybdate (2:1:1 by volume) and, thereafter, 100 μL of 1.0 mol/L citric acid were added to 400 μL of the suitably diluted hydrolysis mixtures or to the mixtures of the phytase assay. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To quantify the released phosphate a calibration curve was produced over 5–600 nmol phosphate.

Statistical methods

The Student’s t test was used for statistical comparison.

Results

Intermediates of enzymatic myo-inositol hexakisphosphate dephosphorylation

Identification of the hydrolysis products of myo-inositol hexakisphosphate generated by Phys was performed by HPIC analysis. The chromatographic profile of the zero-time control indicated only the myo-inositol hexakisphosphate peak (Fig. 2A). After 30 min of incubation, the quantity of myo-inositol hexakisphosphate had decreased...
Table 1. Kinetic constants for the enzymatic dephosphorylation of myo-inositol pentakisphosphate (IP₅).

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<th>IP₅ generated by</th>
<th>IP₅ generated by</th>
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<tr>
<td></td>
<td>the A. niger</td>
<td>the BPP under</td>
</tr>
<tr>
<td></td>
<td>phytase</td>
<td>investigation</td>
</tr>
<tr>
<td>Shewanella oneidensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kₘ (μmol/L)</td>
<td>102±4</td>
<td>107±8</td>
</tr>
<tr>
<td>k₉ (s⁻¹)</td>
<td>156±3</td>
<td>149±4</td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens ATCC 15841</td>
<td>505±17</td>
<td>508±22</td>
</tr>
<tr>
<td>Kₘ (μmol/L)</td>
<td>17.2±0.5</td>
<td>16.9±0.3</td>
</tr>
<tr>
<td>A. niger</td>
<td>132±10</td>
<td>142±19</td>
</tr>
<tr>
<td>k₉ (s⁻¹)</td>
<td>155±11</td>
<td>159±13</td>
</tr>
</tbody>
</table>

Note: The data are means ± SD of five independent experiments. Values were not found to be significantly different (P < 0.05). BPP, β-propeller phytase.

and d/l-Ins(1,2,4,5,6)P₅ appeared as the major degradation product, accompanied by some amounts of Ins(2,4,5,6)P₄. After 60 min of incubation, myo-inositol hexakisphosphate and the myo-inositol pentakisphosphate were completely degraded to Ins(2,4,5,6)P₄ and a myo-inositol trisphosphate which co-eluted with Ins(2,4,6)P₃, d/l-Ins(2,4,5)P₃, and d/l-Ins(1,2,4)P₃. After 90 min of incubation only the myo-inositol trisphosphate peak remained. Prolonged incubation times and increasing enzyme concentration did not result in any appearance of myo-inositol phosphates with less than three phosphate residues.

Identification of the absolute configuration of the generated myo-inositol pentakisphosphate isomer

To determine the absolute configuration of the myo-inositol pentakisphosphate isomer generated by PhyS, kinetic studies with the purified myo-inositol pentakisphosphate isomers generated either by the A. niger enzyme or the phytase from S. oneidensis were performed. The enzymes were added to sequentially diluted solutions of the purified myo-inositol pentakisphosphate isomers and the kinetic parameters (Kₘ, vₘₐₓ) were calculated from the Lineweaver–Burk plots of the data (Table 1). For each individual enzyme, the Kₘ and k₉ for the myo-inositol pentakisphosphate dephosphorylation reactions were almost identical, irrespective of the source of the myo-inositol pentakisphosphate isomers. Thus, these myo-inositol pentakisphosphates are identical. Since it is known that the A. niger phytase predominantly generates the d-Ins(1,2,4,5,6)P₅ isomer (Ullah and Phillips 1988), d-Ins(1,2,4,5,6)P₅ is the first degradation product of myo-inositol hexakisphosphate dephosphorylation by the phytase from S. oneidensis.

Identity of the myo-inositol trisphosphate isomer product

A clear identification of the myo-inositol trisphosphate isomer could not be obtained by HPIC since not all theoretically existing isomers were available. The sole myo-inositol tetraakisphosphate intermediate formed by PhyS (Ins(2,4,5,6)P₄) may be degraded to d/l-Ins(2,4,5)P₃, Ins(2,4,6)P₃, and Ins(4,5,6)P₃. According to HPIC, Ins(4,5,6)P₃ had to be excluded as an intermediate, since this myo-inositol phosphate eluted well resolved from the IP₅ peak generated by PhyS (Fig. 2A). To get more information on the myo-inositol trisphosphate produced by PhyS, the myo-inositol trisphosphate was purified and incubated with the phytases of E. coli and K. terrigena. Both enzymes showed no activity towards the myo-inositol trisphosphate generated by PhyS. The data obtained are consistent with the assumption that Ins(2,4,6)P₃ is the end product of phytate dephosphorylation by PhyS. Escherichia coli phytase is capable of dephosphorylating d-Ins(2,4,5)P₃ (Greiner et al. 2000) and that Ins(2,4,6)P₃ is not a substrate of the K. terrigena phytase (Greiner and Carlsson 2006). Thus, the BPP from S. oneidensis prefers, in contrast to the histidine acid phytases, the hydrolysis of every second phosphate over that of adjacent ones.

Pathway of phytate degradation by Bacillus phytases

The presence of at least one additional phosphatase in the phytase preparation used for the elucidation of the enzymatic phytate degradation pathway might be one reason for the observed discrepancy between the previous model of phytate degradation by Bacillus phytases established by Greiner et al. (2002) (Fig. 1) and the structural computer model of phytate binding (Shin et al. 2001). Since it was shown previously that the phytase preparation used for pathway elucidation were apparently homogeneous according to denaturing and nondenaturing polyacrylamide gel electrophoresis, heat treatment was used to determine whether one or several enzymes were responsible for the hydrolysis of phytate and partially phosphorylated myo-inositol phosphates. The Bacillus phytases purified in this study were partially inactivated by heat treatment and the residual activities toward phytate, ATP, pyrophosphate, and d-Ins(1,2,4,5,6)P₃ were measured to determine if the activities were separable. Heat treatment resulted in no significant difference in loss of activity toward the four phosphorylated compounds studied (Table 2), thus establishing the homogeneity of the enzyme preparations. However, the enzyme preparations used in the previous work (Greiner et al. 2002) were clearly shown to lose significantly higher activity toward phytate compared to ATP, pyrophosphate, and d-Ins(1,2,4,5,6)P₃ (Table 2). In addition, the enzyme preparation used in the previous work was capable of hydrolyzing ATP, whereas the enzyme preparation used in this study did not accept ATP as a substrate. Thus, it can be concluded that these phytase preparations contained at least one additional phosphatase as an impurity.

The highly purified Bacillus phytase preparations were used for phytate degradation studies. The chromatographic profiles obtained during phytate dephosphorylation were indistinguishable for all of these phytases. Therefore, phytate degradation by the phytase from B. amyloliquefaciens ATCC 15841 is shown as an example in Fig. 2B. Combination of HPLC analysis and kinetic studies (Table 1) identified the partially phosphorylated myo-inositol phosphate products as d-Ins(1,2,4,5,6)P₃, Ins(2,4,5,6)P₄, and Ins(2,4,6)P₃. No other phytate dephosphorylation products could be identified even after prolonged incubation times in the presence of high enzyme concentrations.
Table 2. Residual activity toward various phosphorylated compounds after heat treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phytate</th>
<th>d-Ins(1,2,4,5,6)Ps</th>
<th>Pyrophosphate</th>
<th>ATP</th>
</tr>
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<tbody>
<tr>
<td>Bacillus amyloydificaciens ATCC 15841 phytase preparation used in this study</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>126.4±10.3 (100)</td>
<td>97.7±6.8 (100)</td>
<td>6.2±0.4 (100)</td>
<td>0</td>
</tr>
<tr>
<td>10 min at 80 °C with Ca²⁺</td>
<td>63.6±4.7 (50.3)</td>
<td>49.5±3.9 (50.7)</td>
<td>3.0±0.2 (48.4)</td>
<td>nd</td>
</tr>
<tr>
<td>10 min at 80 °C without Ca²⁺</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>nd</td>
</tr>
<tr>
<td>10 min at 100 °C with Ca²⁺</td>
<td>23.1±1.8 (18.3)</td>
<td>17.6±1.3 (18.0)</td>
<td>1.2±0.1 (19.4)</td>
<td>nd</td>
</tr>
</tbody>
</table>

| Bacillus amyloydificaciens ATCC 15841 phytase preparation used in Greiner et al. (2002) | | | | |
| Control | 139.1±10.7 (100) | 13.9±14.3 (100) | 14.0±1.1 (100) | 190.8±12.8 (100) |
| 10 min at 80 °C with Ca²⁺ | 74.0±5.2 (53.2) | 141.5±9.7 (66.1) | 9.5±0.8 (67.9) | 148.7±9.3 (77.9) |
| 10 min at 80 °C without Ca²⁺ | 0 (0) | 80.8±5.2 (37.8) | 5.4±0.4 (38.6) | 143.8±8.7 (75.4) |
| 10 min at 100 °C with Ca²⁺ | 25.9±1.9 (18.6) | 51.7±3.2 (24.2) | 3.5±0.2 (25.0) | 53.7±4.1 (28.1) |

Note: The data are means ± SD of five independent experiments. nd, not determined.

Discussion

Two phosphate binding sites have been identified in the active site of Bacillus phytases (Shin et al. 2001); the “cleavage site,” where hydrolysis of the phytate phosphate takes place, and the “affinity site,” which increases the binding affinity for substrates containing adjacent phosphate groups. The existence of two nonequivalent phosphate binding sites points to a preferred hydrolysis of every second phosphate group over that of adjacent ones and the generation of a myo-inositol trisphosphate as the final product of phytate degradation. Superposition of phytate into the active site suggested that only the phos- phate groups at position C-3 and C-6 are accessible for cleavage by the Bacillus phytases and phytate degradation should proceed either via Ins(2,4,5,6)P₄ to Ins(2,4,6)P₃ or via Ins(1,2,3,5)P₄ to Ins(1,3,5)P₃ (Shin et al. 2001). However, phytate degradation studies only identified d-Ins(1,2,4,5,6)P₅ (Greiner et al. 2002) or d/L-Ins(1,2,4,5,6)P₅ (Kerovuo et al. 2000) as the first intermediate (Fig. 1). Both studies suggested a dual pathway of enzymatic phytate hydrolysis. The pathway given by Kerovuo et al. (2000) fits with the structural computer model, but d-Ins(1,2,3,4,5)P₅ did not accumulate during phytate hydrolysis and Ins(1,3,5)P₃ was only found in small amount. The degradation pathway given by Greiner et al. (2002) fits only partly with the structural computer model since removal of the phosphate residue at C-4 of d-Ins(1,2,3,4,5)P₅ is not consistent with the preferred hydrolysis of every second phosphate group. Heat treatment studies showed that there was extra phosphatase activity in the enzyme preparation previously used by Greiner et al. (2002). In this study a higher quality enzyme preparation was used by an indirect method, which was a comparison of the sensitivity of their products towards another class of phytase.

In summary, no dual phytate degradation pathway could be established for the BPPs as reported previously by Kerovuo et al. (2000) and Greiner et al. (2002). The following four reasons point to the correctness of the phytate degradation pathway given here. First, all three studies on phytate dephosphorylation by BPPs reveal Ins(2,4,6)P₃ as a final dephosphorylation product. Second, heat treatment studies point to the absence of additional phosphatase activity in the Bacillus phytase preparations. Third, in this study as well as in our previous one (Greiner et al. 2002), Ins(1,3,5)P₃ is not detected as a final degradation product of the BPPs as reported by Kerovuo et al. (2000). The formation of Ins(1,3,5)P₃ upon action on phytate would require hydrolysis of the phosphate residue at position C-2 of the myo-inositol ring. This phosphate residue was shown to be resistant to dephosphorylation by phytases (Konietzny and Greiner 2002). Furthermore, phytases have been shown to remove phosphate stepwise from the phytate molecule, whereby each myo-inositol intermediate is released from the enzyme and may become a substrate for further hydrolysis (Konietzny and Greiner 2002). However, Kerovuo et al. (2000) were unable to identify d/L-Ins(1,2,4,5,6)P₅ as an intermediate of phytate dephosphorylation by a Bacillus phytase. In addition, Ins(1,3,5)P₃ did not accumulate to the same extent as Ins(2,4,5,6)P₄ (Kerovuo et al. 2000). The reason for the appearance of an additional myo-inositol trisphosphate in the study of Kerovuo et al. (2000) is still unclear. Finally, the phytate degradation pathway is consistent with the structural data and results of a substrate recognition model (Shin et al. 2001).

References


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